United States Environmental Protection Agency

LABORATORY ANIMAL PROJECT REVIEW

Please note:

1. All information in this LAPR is considered privileged and confidential by the IACUC and regulatory authorities.
2. Approved LAPRs are subject to release to the public under the Freedom of Information Act (FOIA). Do not include proprietary or classified information in the LAPR.
3. An approved LAPR is valid for three years.

LAPR Information

LAPR Title:	**Cylindrospermopsin-induced bleeding in mice: evaluating mechanisms and its role in toxicity.**
LAPR Number:	**17-05-005**
Principal Investigator	**Exemption 6**
Author of this Document:	**Exemption 6/RTP/USEPA/US**
Date Originated:	**04/21/2011**
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Date Approved:	**06/13/2014**
Date Closed:	**06/02/2017**

APPROVALS

APPROVER	NAME	APPROVAL DATE	COMMENTS	
	Exemption 6 Exemption 6 Exemption 6/RTP/USEPA/US by Exemption 6 /RTP/USEPA/US	06/13/2014	DMR	
	Exemption 6/RTP/USEPA/US by Exemption 6/RTP/USEPA/US	06/13/2014		

Administrative Information

1. Project Title (no abbreviations, include species):
Cylindrospermopsin-induced bleeding in mice: evaluating mechanisms and its role in toxicity.

Is this a continuing study with a previously approved LAPR?
Yes
Please provide the previous LAPR# 14-08-002

2. What is the Intramural Research Protocol (IRP) number covering this project?
IRP-NHEERL-TAD/DBB[redacted]-11-16-010 SSWR 2.3c

3. EPA Principal Investigator/Responsible Employee:

Principal Investigator	*Phone Number*	*Division*	*Mail Drop*
Exemption 6	Exemption 6 *Lotus Notes Address* [redacted]Exemption 6/RTP/USEPA/US	TAD *Branch* DTB	MD 67

4. Alternate Contact:

Alternate Contact	*Phone Number*	*Division*	*Mail Drop*
Exemption 6	Exemption 6 *Lotus Notes Address* [redacted]TP/USEPA/US	TAD *Branch* DTB	MD 67

SECTION A - Description of Project

1. Study objectives, presented in <u>non-technical language</u> such that it is understandable by non-scientific persons, including how the study addresses health protection. If this is a continuing study from a previous LAPR, briefly justify the continuation. Please spell out all acronyms and abbreviations with their initial use.
This LAPR is designed to test a hypothesis concerning the mechanism of action responsible for the bleeding induced in people and laboratory mice by the cyanobacterial toxin, cylindrospermopsin (CYN).

Cyanobacteria are photosynthetic bacteria found in all bodies of water and capable of adversely affecting water quality. When environmental conditions are optimal, massive rapid growth ("blooms") may result. Many cyanobacterial species produce toxins that are proven threats to human health and hazardous algal blooms are an increasingly serious national and international problem as frequency and geographical range increases. Cyanotoxins have, in the last few years, caused deaths of dogs, and human illness after exposures via recreational waters in California, Montana, and Ohio. The potential exposure to cyanotoxins through consumption of fish and assorted invertebrates is also a realistic scenario since significant bioaccumulation of these toxins has been demonstrated in numerous studies. Cyanobacterial toxins (microcystin-LR, anatoxin-a, CYN) are on the 3rd Federal Drinking Water Contaminant Candidate List (CCL3) and are monitored as required under the EPA's Unregulated Contaminant Monitoring Guidelines.

One of these toxins, CYN, has caused adverse human health effects after ingestion of chlorinated drinking water supplied to municipalities. It has been identified in many freshwater bodies in the United States including both Falls Lake and Jordan Lake. It is produced by at least one common freshwater cyanobacterial species, Aphanizomenon flos-aquae. In recent years, another freshwater species that is known to produce CYN in both Australia and Mexico, Cylindrospermopsis raciborskii, has become the primary phytoplanktonic species in north-central Florida, accounting for over 95% of the phytoplankton for most of the year in numerous lakes in that region. With both the ease of transport of dormant cells that are able to survive harsh conditions for extremely long periods of time, and the potential of transfer of CYN-producing genes between species, there is a possibility of C. raciborskii eventually producing CYN in US waters. Both C. raciborskii and A. flos-aquae bloom 3-4 meters beneath the water surface and are often in close proximity to intake pipes used to obtain water for drinking. These species have no unusual taste, color, or odor. The combination of these traits makes both species difficult to locate until a bloom has passed. The toxin, unlike most other cyanobacterial toxins, is freely excreted by the organisms and is therefore present in the water column both during and after the life of the bloom.

Bleeding is a prominent symptom of CYN toxicity. An incident in the town of Palm Island, Australia, resulted in the severe illness and hospitalization of 140 people (mostly children) who were exposed to CYN via treated drinking water. The initial symptoms included vomiting, anorexia, constipation, enlarged and tender liver (hepatomegaly), and general malaise. The symptoms progressed to a point where hospitalization was necessary and included blood in the urine, bleeding mucous membranes, and profuse bloody diarrhea (40% of patients) that persisted for up to three weeks. Identifying the cause(s) of the bleeding is important because knowledge of the basic mechanism(s) of bleeding may lead to better treatment regimens in the event of another poisoning event.

The mouse is known to be an excellent model to simulate the toxin’s known adverse effects on humans. We and others have shown that exposure to CYN produces many of the effects seen in humans including bleeding. In the mouse we observed blood-filled gastrointestinal tracts (20% of the animals), and bleeding in the tail tips (30%), peri-orbital sinus (3%), and testes. Related to this issue is our finding that CYN induces premature birth (an extremely rare phenomenon in rodents). This finding may be due to the placental bleeding at the maternal-fetal interface that we have noted in preliminary histological analysis. Bleeding in this area often results in a separation of the fetal and maternal portions of the placenta (abruptio placentae) and is known to induce human miscarriage and premature birth.

Data indicate that the toxic effects of CYN may be due to the general bleeding taking place in capillary-rich areas. Last year, we sent blood samples of treated and control animals to a commercial laboratory (Antech) specializing in the evaluation of coagulation parameters. The results clearly indicated that CYN is a potent anti-coagulant, extending prothrombin time (PT) and partial thromboplastin time (PTT) beyond the limits of the test system. Anti-coagulation effects were seen after a single dose in a small percentage of animals but were present in almost all animals after three consecutive daily doses. PT and PTT values of treated animals returned to normal six days post-dosing.

Our previous studies with CYN have demonstrated that it was not primarily a hepatic toxin as had been generally accepted. We had identified anti-coagulant activity as a possible mechanism. In these studies, two genes that are critical for the proper functioning of vitamin K in the coagulation cascade are significantly under-expressed in the liver soon after CYN dosing and we hypothesize that lack of active vitamin K may be the cause of CYN-induced bleeding. In veterinary practice, vitamin K is used to treat animals after exposure to warfarin-like rodenticides that affect the metabolism of the vitamin and cause fatal bleeding. Similarly, we will see if vitamin K

protects mice from CYN's effects. We will use warfarin as a positive control to determine the dose of vitamin K sufficient to protect a warfarin-exposed animal from its anti-coagulant effects. PT and PTT will be used as a measurement of anticoagulant activity.

There are four objectives of studies in this LAPR:
A. Instrument data validation.
We have obtained a Stago Instrument that measures PT (prothrombin time) and PTT (partial thromboplastin time) which are the two main tests to evaluate the general status of the coagulation process. The instrument also measures a variety of blood coagulation factor proteins. The initial objective of this LAPR is to familiarize ourselves with the Instrument and obtain sufficient consistent data reflecting expected values from untreated mice to be sure that subsequent data will accurately reflect coagulation system status.

B. Dose-finding studies.
CYN - to determine a single appropriate dose that will reliably prolong PT and PTT.
Warfarin – to determine a dosing regimen (preferably a single dose) that will reliably prolong PT and PTT.
Vitamin K – to determine a dosing regimen that will protect against warfarin-induced effects on PT and PTT. This Vitamin K dose will then be used in combination with CYN exposure (see "D" below).

C. Time-course studies.
We will follow the course of the anti-coagulant activity in male, female, and pregnant female animals as well as exposed fetuses and pups. Endpoints will consist of clinical signs; PT and PTT values; coagulation factor gene and protein levels; changes in the expression of genes that are essential for the coagulation process; histopathology of organ systems and tissues that are affected by CYN; and the pattern of anti-coagulation in fetuses and newborn pups as measured by clinical signs and gene expression.

D. CYN and Vitamin K protection studies.
a. Vitamin K pre-CYN treatment as a possible protection against induced anti-coagulation.
b. Vitamin K post-CYN treatment as possible remedy for induced anti-coagulation.

NOTE: We do not anticipate overt toxicity with the dosing regimens we will be using; however, because of the potential for severe effects, all animals exposed to CYN or warfarin will be considered Category E.

2. Scientific rationale for proposed animal use.

a. Why is the use of animals necessary?

There is no computer model or in vitro bioassay system that can accurately predict anti-coagulation effects. There are no validated in vitro sytems that can be used to evaluate anti-coagulation effects in an intact animal. The target system(s) of CYN remains unknown as does its pharmacokinetics and the possibility of active metabolites. These considerations lead to the conclusion that an in vivo approach is the only feasible research strategy to obtain answers to the questions we are asking in this Proposal.

b. Justify the species requested:

Our previous studies with the mouse have shown that the i.p. route of administration results in a syndrome of effects that closely resembles those seen in humans exposed to this toxicant as well as other possible effects (e.g. placental bleeding, fetal toxicity) that were not reported in the toxicity episode.

3. How was it determined that this study is not unnecessary duplication?

A literature search using Medline and Toxline (1986-present) on cylindrospermopsin was done and no studies were identified that directly addressed bleeding as the primary factor in CYN-induced toxicity. To date, there have been no other hypotheses put forward, and CYN is now simply referred to as a "cytotoxin" with an unidentified mechanism of action.

SECTION B - In Vivo Procedures

1. Briefly describe experimental design. Supplementary information may be attached at the end of the LAPR, but please include critical information within the body of the LAPR.

There are four objectives of studies in this LAPR:

A. Instrument data validation.

The entire study depends on our ability to obtain accurate coagulation data. We know what normal PT, and PTT values are as well as normal circulating levels of key coagulation factors. These normal values are being obtained from the Antech data on CYN coagulopathy, literature values, and data obtained by veterinarians studying mouse coagulopathy at the North Carolina State University College of Veterinary Medicine. The analyses will be done on a Stago blood analysis instrument that we have purchased. We will be trained on the instrument by a Company representative but it is essential that we are confident of the accuracy and consistency of the values we obtain prior to initiating the remaining studies. We will be using young adult animals for these analyses: 20 males, 20 non-pregnant females, and 10 pregnant females.

B. Dose-finding studies.

CYN

The studies on CYN anti-coagulation are based on the identification of a dose level that will significantly prolong PT and PTT and determine the onset of the anti-coagulation effect and its duration. Our data indicate that a single i.p. dose of CYN produces a significant prolongation of the PT and PTT times 24 hours post dosing with 50ug/kg, but the effect only occurred in 2/12 animals. After two 50ug/kg doses given 24hrs apart, the effect was seen in 6/8 animals. Optimally, we want to use a single dose of CYN that will produce anti-coagulation in most animals so that we will be able to test the course of the effect without the complexity of multiple doses that cause the effect over a longer period of time. The endpoint for this Section of the study is the determination of the mechanism rather than extrapolation of the data to human populations. We propose to do a dose-response study beginning at 100ug/kg (based on an assumption that CYN exposure produces cumulative toxicity) and measuring the degree of anti-coagulation by PT and PTT times. It is impossible to predict whether the 100ug dose level will be sufficient to do this so we are asking for the dose range that can be tested to go as high as 250ug/kg. We will begin with the 100ug dose level and increase levels only if 100ug/kg is not effective to produce a significant anti-coagulation effect in ≥ 75% of the treated animals. We are proposing to purchase 48 mice (24 males and 24 non-pregnant females) to run 4 dose finding experiments (if needed) with CYN to pinpoint the desired dose. There will be 6 mice/sex (2 control + 4 treated) x 2 sexes x 4 experiments = 48 mice [16 Category C, 32 Category E]. Animals will be ordered as needed.

Warfarin

We wish to determine a dosing regimen that will prolong PT and PTT. The general design outlined immediately above will be used for the warfarin dose finding study. Our objective is to determine the dose of warfarin that prolongs PT and PTT ≥ a 4-fold increase in ≥ 75% of the animals. An initial dose of 3mg/kg warfarin will be used, based on literature citing its use as a positive control to prolong PT and PTT. It will be administered by gavage in sterile water. 24hrs post-dosing, animals will be euthanized and the PT/PTT times determined. The dose of warfarin will be increased, if necessary, to 6mg/kg. We are proposing to purchase 48 mice (24 males and 24 non-pregnant females) to run 4 dose finding experiments (if needed) with warfarin to determine the active dose. There will be 6 mice/sex (2 control + 4 treated) x 2 sexes x 4 experiments = 48 mice [16 Category C, 32 Category E]. Animals will be ordered as needed.

Vitamin K

We wish to determine a dosing regimen that will protect against warfarin-induced effects on PT and PTT so that we will know that the dose of vitamin K used in the CYN-vitamin K studies (see "D" below) is an effective dose against warfarin-related anti-coagulation. The general design will be similar to the protocols used to determine the effective doses of CYN and warfarin outlined immediately above. We will use the dose of warfarin that was determined immediately above and test the effects of different dose levels of vitamin K, initially 2mg/kg administered in 0.1ml corn oil by gavage on two consecutive days to determine if it prevents the warfarin-induced anti-coagulation (prolonged PT and PTT times). If the initial dose of vitamin K is not effective we will increase the dose to a maximum of 5mg/kg. The following day animals will be euthanized and PT/PTT times determined. There will be 12 mice/sex (2 control, + 2 warfarin, + 6 vitamin K + warfarin + 2 vitamin K alone) x 2 sexes x 4 experiments = 96 mice [32 Category C, 64 Category E]. Animals will be ordered as needed.

C. Time-course studies of CYN-induced anti-coagulation.

Young sexually mature, male, non-pregnant female, and pregnant female CD-1 mice will be dosed once with CYN at the determined effective single dose, i.p., in sterile water in a volume of 0.1ml (pregnant females will be dosed on Gestation Day (GD) 16).
The course of the anti-coagulant effect of CYN at the effective dose determined in Section B above, will be monitored over a 6-day time period at a total of 10 intervals that will allow us to assess the onset and persistance of the full effect and the subsequent recovery from the effect. Animals will be euthanized at 1, 3, 6, 12, 24, and 48 hours (2 days), 3 days, 4 days, 5 days, and 6 days after dosing. 12 non-pregnant treated animals (6 males and 6 females) and 4 controls (2 males and 2 females) will be euthanized at each time point. Animals will be monitored for clinical signs of toxicity. After euthanasia, blood will be obtained for analysis for PT/PTT and levels of FX coagulation factor protein. Tissues including liver, tail tips, gastrointestinal tract (GIT), blood vessels, pancreas, and testes will be collected for histological evaluation. Groups of 6 pregnant animals (4 treated and 2 control) will be euthanized on GD17, PND2 and PND20 and, in addition to the gene and histology endpoints listed above, we will collect fetuses and pups and do critical coagulation gene (Vkorc1, Ggcx) analyses on liver tissue from maternal and fetal/PND2 animals.
The total animals needed for this part of the project are 80 males (20 control and 60 treated), 80 non-pregnant females (20 control and 60 treated) and 18 pregnant females 6 control and 12 treated) - a total of 178 animals (46 controls and 132 treated). Twelve of these pregnant females will produce litters averaging 12/pups/litter (48 controls and 96 treated). A flow chart outlining the course of this aspect of this study objective is attached.

D. CYN + Vitamin K protection studies (all animal numbers are equal males and females).

a. Vitamin K treatment as possible protection against onset of CYN-induced anti-coagulation.

The protective effect of vitamin K involve the administration of the previously determined effective vitamin K dose (see Section B) by gavage in 0.1ml corn oil one day prior to dosing and a similar amount of vitamin K on the following day at the same time as animals will be dosed with the effective anti-coagulant dose of CYN to determine if vitamin K can lessen the coagulation-related CYN toxicity. Concurrent vitamin K and CYN positive controls will receive the doses of vitamin K in 0.1ml corn oil by gavage and CYN in 0.1ml sterile water by i.p. injection. Concurrent vehicle controls will receive 0.1ml. sterile water by gavage. Euthanasia and blood collections will be taken at 6, 12, 24 and 48 hours after CYN dosing. Endpoints will include clinical signs of toxicity, gene expression in liver, PT, PTT values and levels of Coagulation Factor 10 in serum. The experimental groups will consist of 4 vehicle controls; 8 vitamin K alone; 12 CYN; and 12 CYN + vitamin K at each of the 4 time points.
The total number of animals for this part of the study will be 144 (48 CYN alone; 48 CYN + vitamin K; 32 vitamin K; and 16 vehicle alone).

b. Vitamin K post-CYN treatment as possible remedy for induced anti-coagulation.

The potential of vitamin K to restore normal coagulation to CYN-treated animals will be tested by following CYN exposure with vitamin K treatment 12 and 24 hours after the average onset time for anti-coagulation as determined in Objective C above. The design of the study will involve 60 animals (30 of each sex) given CYN at a dose determined in the dose finding CYN study in Section B above, and an additional 20 animals used in non-CYN control groups. An initial euthanasia will take place 12hrs after dosing and 12 CYN and 4 control animals (males and females) will be examined at that time to confirm CYN-induced prolongation of PT and PTT times. Once an anti-coagulant effect has been confirmed, 24 (12 males and 12 females) of the remaining 48 CYN-dosed animals will receive the determined dose of vitamin K; 8 animals will receive vitamin K alone; 8 will receive vehicle alone. At 24 hrs after vitamin K dosing, half the animals will be euthanized (groups of 12 CYN alone; 12 CYN + vitamin K; 4 vitamin K alone and 4 vehicle alone). The remaining animals dosed with vitamin K will receive a second dose of vitamin K. All remaining animals will be euthanized 24 hours after the second dose of vitamin K. These animal will consist of 12 CYN alone, 12 CYN + vitamin K; 4 vitamin K alone and 4 vehicle alone. Clinical signs, PT, PTT and gene expression will be obtained from all animals.
The total number of animals for this part of the study will be is 80 (36 CYN alone; 24 CYN + vitamin K; 8 vitamin K; and 12 vehicle alone).

The total number of adult animals for the entire LAPR is 644 (308 males, 308 non-pregnant females, and 28 pregnant females) plus an estimated 144 pups.

2. Justify the number of animals. Include explanation (e.g., biological, statistical, regulatory rationale) for the number of animals needed for each treatment group, and the overall number requested for the duration of the LAPR.

Based on limited data available, we expect that the numbers of animals we are using will allow us to discern reliable differences between different treatment groups as well as between treatment and control groups.
The numbers of animals used are:
Section A - Instrument data validation: 20 males, 20 non-pregnant females; 10 pregnant females all 50 Category C
Section B - CYN Dose-Finding: 8 males and 8 females Category C: 16 males and 16 females Category E
Section B - Warfarin Dose-Finding: 8 males and 8 females Category C: 16 males and 16 females Category E
Section B - Vitamin K Dose Finding: 16 males, 16 females Category C; 32 males; 32 females Category E
Section C - Time Course of CYN anti-coagulation: 20 males, 20 non-pregnant females, 6 pregnant females Category C, 48 pups Category C, 60 males, 60 non-pregnant females; 12 pregnant females Category E, 96 pups Category E
Section D.a. - Vitamin K Protection: 24 males and 24 females Category C; 48 males and 48 females Category E.
Section D.b. - Vitamin K treatment: 10 males and 10 females Catefory C; 30 males and 30 females Category E.

3. State how many animals over the study period are expected to be used under the following three categories of pain/distress (USDA nomenclature as defined in the instructions): Please enter numbers only.

Categories	Adults	Offspring
C) Minimal, transient, or no pain/distress:	228	48
D) Potential pain/distress relieved by appropriate measures:		
E) Unrelieved pain/distress:	416	96

4. For tracking purposes, please check if this LAPR includes any of the following:

☐ Restraint (>15 Minutes) ☐ Survival surgery
☐ Food and/or water restriction (>6 Hours) ☐ Non-survival surgery

5. Category C procedures. Describe each procedure separately, include details on the following:

a. Treatments (e.g., dosages, duration of exposure, route, volume, frequency):
Controls willl be given a single i.p. injection of 0.1ml sterile water concurrently with CYN dosage. All animals will be dosed 6 days after arrival at the animal facility. Pregnant animals will be dosed on gestation day 16. Pharmaceutical grade Vitamin K will be administered at a maximum of two doses of 5mg/kg by gavage in 0.1ml corn oil/dose.

b. Blood collection (method, volume, frequency):
Blood will be collected at the time of euthanasia.

c. Testing methods (including non-stressful dietary restrictions/modifications, mild non-damaging electric shock):
na

d. Animal restraint and confinement beyond routine housing and handling. Include a description of the type of restraint device, acclimation to device, duration of restraint:
na

e. Breeding for experimental purposes (e.g. length of pairing, number of generations):
na

f. Describe how animals will be monitored (e.g., frequency of observations, by whom):
Animals will be monitored three times on the day of dosing and the subsequent two days by laboratory staff. After that, animals will be monitored twice daily.

6. Non-surgical Category D or E procedures. Describe each procedure separately, include details on the following (Also fill in Section B.9).

a. Treatments (e.g. dosages, duration of exposure, route, volume, frequency):

CYN will be given as a single dose at a maximum of 250ug/kg. All dosing will be by the i.p. route in 0.1ml sterile water. All animals will be dosed at least 3 days after arrival at the animal facility. Pregnant animals will be dosed on gestation day 16.
Warfarin will be given by gavage at a maximum single dose of 6mg/kg in sterile water.

b. Blood collection (method, volume, frequency):
Blood collections will be done as exsanguination at the time of euthanasia.

c. Testing methods:
All testing will be done at the time of euthanasia when target tissues and blood will be collected.

d. Restrictions placed on the animals' basic needs (e.g., food and/or water deprivation, light cycles). Provide details regarding the length of deprivation:
na

e. Describe how animals will be monitored (e.g., frequency of observations, by whom):
3 times daily for two days following CYN dosing and twice daily thereafter.

f. Analgesia (Category D Procedures) - list drugs, dosages, route of administration and frequency:
na

g. If treatment-related deaths are expected, this must be thoroughly justified. Death as an endpoint is highly discouraged:
no deaths are expected

7. Surgical Category D and E procedures. Describe each procedure separately, include details on the following (Also fill in Section B.9)

a. Complete description of surgical procedure including presurgical preparation, aseptic technique, surgical closure, etc:
na

b. Anesthetic regimen (drugs, dosages, volume, and route of administration). The use of paralytic or neuromuscular blocking agents without anesthesia is prohibited:
na

c. Postoperative care (thermal support, special feeding, frequency and duration of monitoring, responsible personnel, removal of sutures/staples):
na

d. Post operative analgesics (drugs, dosage, and volume and route of administration, frequency):
na

e. Will any animals be subject to more than one major surgical survival procedures?
○ Yes ● No

f. Identify any surgical procedures performed at other institutions or by vendors:
na

8. Humane interventions (for treatments/procedures in all categories).

a. Describe actions to be taken in the event of expected or unexpected deleterious effects from procedures or chemical exposures.
Animals will be euthanized and tissues obtained if possible.

b. State criteria for determining temporary or permanent removal of animals from the study.
Any animals that require euthanasia due to severe bleeding, non-responsiveness to interaction, inappetance, lethargy, and/or hypothermia will be removed from the study, necropsies will be performed and blood and tissues will be collected for analysis as needed.

9. Alternatives to pain and distress (Category D and E Procedures only). Provide narrative regarding the sources consulted to ascertain whether acceptable alternatives exist for potentially painful/distressful procedures. Include databases searched or other sources consulted, the date of the search and years covered by the search, and key words and/or search strategy used. Assistance with searches is available through the EPA Library Staff.
Medline was searched from 1980 to present. Key words were organ function, bleeding, anti-coagulation, and cylindrospermopsin toxicity. There are no validated in vitro sytems that can be used to evaluate anti-coagulation effects in an intact animal. The target system(s) of CYN remains unknown as does its pharmacokinetics. These considerations lead to the conclusion that an in vivo approach is the only feasible

research strategy to obtain answers to the questions we are asking in this Proposal.

SECTION C - Animal requirements

Describe the following animal requirements :

1. Indicate the number of animals required over the study period for this protocol. Please enter numbers only.

a. Animals to be purchased from a Vendor for this study: 644

b. Animals to be transferred from another LAPR:

LAPR Number that is the source of this transfer:

c. Animals to be transferred from another source:

d. Offspring produced onsite (used for data collection and/or weaned): 112

e. TOTAL NUMBER of animals for duration of the LAPR 756

2. Species (limited to one per LAPR): Mouse/Mice

3. Strain: CD-1 mouse/mice

Describe special requirements for animals with altered physiological responses (e.g., genetically altered, aged)

na

4. Sources of animals:

Charles River - Raleigh

5. Provide room numbers where various procedures will be performed on animals:

6. Will any animals be housed in areas other than the animal facility longer than 12 hours? If so, state location. Such areas require prior IACUC approval as a satellite facility before LAPR can be reviewed.

no *Room Numbers:*

7. Describe any transportation and containment methods involved in moving animals between EPA buildings, or between EPA and other institutions (excluding any commercial shipments)

none

8. Describe any unusual housing or husbandry requirements, or acclimation requirements. Justify any treatment beginning less than 3 days after arrival.

none

9. Describe special assistance requested of the animal contract staff, including procedures and dosing. NOTE, this request must be submitted separately to the Animal Resources Program Office (ARPO)

none

10. Housing and Enrichment.

The IACUC encourages the use of environmental enrichment whenever possible (see IACUC website for details). Provide details on how the animals will be housed, including type of cage (e.g., solid bottom or wire screen), bedding material, number of animals per cage, and environmental enrichment. Note that housing rodents individually without environmental enrichment requires justification.

Animals will be housed 4/cage except for pregnant animals that will be housed one/cage upon arrival. Pregnant animals must be housed separately to preserve litter identity and since they will be arriving mid-gestation (Gestation Day (GD) 12) and will be dosed on GD16 they should be housed separately so as not to induce extra stress shortly before parturition. Polycarbonate cages with solid bottom; automatic water delivery system, and pine shavings bedding will be used. Enrichment should be limited to plastic "igloos" (no nestlets).

SECTION D - Hazardous and Non-Hazardous Agents

1. Identify all hazardous and non-hazardous agents to be administered to living animals. For agents requiring a Health and Safety Research Protocol (HSRP), provide the title of the approved HSRP for each such agent. If no protocol is required for an agent deemed potentially hazardous (e.g. nanoparticles, recombinant DNA), describe the safety precautions to be used.
Provide maximum dosing levels and route-appropriate LD50s (where available) for each agent used for dosing.

The HSRP for CYN is "HSRP-164".
Maximum dose 250ug/kg of cylindrospermopsin (CYN), i.p. injection at a single time point. The published 1-day LD50 is above 1000ug/kg i.p.. CYN is not volatile and remains in the feces, urine and carcasses of the animals. It is destroyed during the incineration of the experimental animals and their bedding. The carrier (distilled water) is sterile and the CYN has been made up in sterile distilled water and stored at -20 degrees in the dark.
The maximum dose of pharmaceutical grade warfarin sodium will be 300mg/kg administered by gavage as a positive control to prolong PT and PTT times. The oral LD50 is listed in the Register of Toxic Effects of Chemical Substances as 374mg/kg of warfarin sodium. Dosing will be in food grade corn oil. Vitamin K (pharmaceutical grade) will be administered in food grade corn oil by gavage. Vitamin K has an oral LD50 in the mouse of 25g/kg. It will be adminsitered at maximum dose of 5mg/kg.

2. Describe any plans to administer human or animal tissues, blood or body fluids to the animals in this LAPR, and provide:

a. Information to assure that such material is pathogen-free
na

b. A statement regarding any safety precautions necessary for handling the material.
na

NOTE: Any unresolved health/safety questions which arise during IACUC review of this LAPR will require consultation with the Safety, Health, and Environmental Management Office.

SECTION E - Personnel Training and Experience

1. Identify all project personnel conducting animal experimentation. Specify the techniques for which they have responsibility, and their relevant training and experience. Additional personnel may be added to the table below as a group (by Division) for Category C procedures. By so doing you are giving assurance that these personnel have received all required training and are qualified to perform the Category C techniques requested.
Training & Experience:

NAME	ROLE	SPECIFIC RESPONSIBILITY	RELEVANT TRAINING
Exemption 6	Principal Investigator	Design of study, participation in dosing, weighing, monitoring and necropsy.	40 years of experience in animal toxicology studies. All relevant animal use NHEERL training courses.
Exemption 6	Associate Principal	Design of study, participation in dosing,	Licensed veterinarian. All relevant animal use NHEERL training courses.

	Investigator	weighing, monitoring and necropsy.	
Exemption 6	Post-Doc	Participation in dosing, weighing, monitoring and necropsy.	All relevant animal use NHEERL training courses.
	Tech Support	Category C Procedures	EPA IACUC Trained

SECTION F - Animal Breeding Colonies

This section pertains to the breeding of animals for maintenance of ongoing animal colonies. Do not include breeding that is part of experimentation and accountable under Section C.

Describe:

1. Estimated number of breeding pairs and liveborn per year na

2. Breeding protocols and recordkeeping na

3. Methods for monitoring genetic stability na

4. Disposition of all offspring and retired breeders that are not used in accordance with the procedures described in this LAPR na

SECTION G - Euthanasia

1. When will the animals be euthanized relative to experimental procedures?

2. Describe the euthanasia techniques:

Method(s): Anesthesia plus exsanguination
Agent(s): CO2
Dose (mg/kg): To effect
Volume: Full
Route: Inhalation

Source(s) of information used to select the above agents/methods:

_ Personal Experience, IACUC Pain Euthanasia Guidelines

3. Provide justification and references for any euthanasia agent or method that is not consistent with recommendations of the 2007 American Veterinary Medical Association (AVMA) Guidelines for Euthanasia (e.g., cervical dislocation or decapitation without anesthesia; cervical dislocation in rodents weighing more than 200 grams).

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4. Describe how death is to be confirmed.

Prolonged cessation of breathing

SECTION H - Disposition of Used and Unused Animals

Describe the disposition of any animals remaining after project completion.
Euthanized as above
The IACUC encourages investigators to reduce the overall number of animals used at NHEERL. Would you consider transferring any unused animals from this LAPR to another approved LAPR?
● Yes ○ No

SECTION I - Assurances

1. Animals will not be used in any manner beyond that described in this application without first obtaining formal approval of the IACUC.
2. All individuals involved in this project have access to this application, are aware of all EPA policies on animal care and use, and are appropriately trained and qualified to perform the techniques described.
3. The proposed research using animals does not unnecessarilty duplicate any previous experimentation.
4. Thorough consideration of the three "R"'s (Replacement, Reduction, Refinement) has been given, as applicable, to a. the use of animals, and b. procedures causing pain or distress (with or without analgesia/anesthesia), including death as an endpoint. The minimum number of animals required to obtain valid experimental results will be used.
5. The Attending Veterinarian has been consulted in regard to any planned experimentation involving pain or distress to animals.
6. All procedures involving hazardous agents will be conducted in accordance with practices approved by the Safety, Health, and Environmental Management Office.
7. Individuals from outside of EPA who are collaborating on this project, and who conduct related experimentation on EPA procured or bred animals in their respective Institutions, have the equivalent of a current IACUC approved LAPR at their respective Institutions.
8. The IACUC has oversight responsibilities for animal care and use, and may request consultation or feedback regarding the conduct of in vivo procedures, progress and accomplishments, and any problems encountered.

EPA Principal Investigator	Certification Signature Date
Exemption 6 by Exemption 6	05/14/2014

Submitted: 05/14/2014

Certification:

Certification by EPA Supervisor (Branch Chief or Division Director) that the project described herein has been reviewed and approved on the basis of scientific merit:

Branch Chief/Division Director	*Approval Date*	*Phone Number*	*Division*	*Mail Drop*
Exemption 6	05/14/2014 by Exemption 6 Exemption 6 Exemption 6 RTP/USEPA/US	Exemption 6 *Lotus Notes Address* Exemption 6 Exemption 6 Exemption 6 RTP/USEPA/US	TAD *Branch* DTB	MD *Submitted to Branch Chief for Approval* 05/14/2014 01:13 PM

ATTACHMENTS

17-05-005 PI resp.pdf Summary of LAPR on CYN and Coagulation.final6-10-14.pub

LAPR objectives for CYN bleeding studies - all mice in table.final6-10-14.xlsx

Actions

First Update notification sent: 03/27/2015
Second Update notification sent:
First 2nd Annual notification sent: 04/13/2016
Second 2nd Annual notification sent:

1st Expiration notification sent: 04/03/2017
2nd Expiration notification sent: 05/02/2017

History Log: